Microbiology (2006), **152**, 2651–2659 DOI 10.1099/mic.0.28981-0

Cyt1Ca from *Bacillus thuringiensis* subsp. *israelensis*: production in *Escherichia coli* and comparison of its biological activities with those of other Cyt-like proteins

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Received 9 March 2006
Revised 15 May 2006
Accepted 22 May 2006

The larvicidal activity of *Bacillus thuringiensis* subsp. *israelensis* against dipteran larvae is determined by four major polypeptides of the parasporal crystalline body produced during sporulation. Cyt1Aa shows the lowest toxicity when used alone but is the most synergistic with any of the other proteins. The sequence of the plasmid pBtoxis, which contains all the toxin genes in this subspecies, revealed a new *cyt*-like coding sequence named *cyt1Ca*. In addition to the Cyt-like region, the predicted Cyt1Ca contained an extra domain at the C terminus, which appeared to be a β-trefoil carbohydrate-binding motif, as found in several ricin-like toxins. The gene was PCR-amplified from pBtoxis and cloned in several vectors, allowing high-level expression in *Escherichia coli*. Cyt1Ca was purified by nickel-nitrilotriacetic acid affinity chromatography, characterized, and its biological activity was determined. Toxicity against larvae of *Aedes aegypti* of Cyt1Ca in recombinant *E. coli* cells was compared with that of Cyt1Aa and Cyt2Ba, and the ability of these proteins to enhance the activity of Cry4Aa was assessed. Although Cyt2Ba appeared able to interact with Cry4Aa, no activity for Cyt1Ca was observed, even when produced in truncated form. Furthermore, in contrast to Cyt1Aa, Cyt1Ca did not lyse sheep erythrocytes, and it was not bactericidal to the host cell.

INTRODUCTION

During sporulation, various subspecies of the Gram-positive bacterium *Bacillus thuringiensis* produce large amounts of insecticidal crystal proteins (ICPs), the so-called δ-endotoxins (Schnepf *et al.*, 1998), which are toxic against larvae of diverse groups of insects. These proteins belong to two unrelated families: receptor-specific Cry toxins, active against insects, and Cyt toxins that lyse a broad range of cells, including bacteria, by binding to phospholipids. The ICPs of *B. thuringiensis* subsp. *israelensis* are specific against larvae of mosquitoes and black flies (Goldberg & Margalit, 1977), and are composed of four major polypeptides, Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa, encoded by respective genes located on the 128 kb plasmid pBtoxis (Berry *et al.*, 2002). Cyt1Aa is the most prominent and least specific toxin, showing haemolytic and cytotoxic activities *in vitro* (Thomas & Ellar, 1983; Hofte & Whiteley, 1989). Its mosquito larvicidal activity is low, but it acts synergistically to potentiate the activity of Cry4Aa, Cry4Ba or Cry11Aa (Wu *et al.*, 1994; Crickmore *et al.*, 1995). These Cyt/Cry synergies are significantly greater than any synergistic interactions between the Cry toxins themselves (Crickmore *et al.*, 1995; Khasdan *et al.*, 2001). The role of Cyt1Aa in retarding resistance to the Cry proteins is crucial (Wirth *et al.*, 1997, 2005). In addition, recombinant *Escherichia coli* (Douek *et al.*, 1992) loses its colony-forming ability upon expressing Cyt1Aa, a lethal action that is circumvented by the accessory protein P20 (Manasherob *et al.*, 2001).

Seven cytolytic toxins (Cyt1Aa, -1Ab, -1Ba, -2Aa, -2Ba, -2Bb and -2Bc) have been characterized in mosquitocidal subspecies of *B. thuringiensis* (Waalwijk *et al.*, 1985; Thiery *et al.*, 1997; Delécluse *et al.*, 2000; Koni & Ellar, 1993; Guerchicoff *et al.*, 1997; Cheong & Gill, 1997; Juárez-Pérez *et al.*, 2002), the most studied of which is Cyt1Aa (Margalith & Ben-Dov, 2000), but the crystal structure of Cyt2Aa from *B. thuringiensis* subsp. *kyushuensis* is the only one elucidated to

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date (Li *et al.*, 1996). Since Cyt1Aa is 38 % identical to Cyt2Aa (with many of the amino acid differences being conservative in nature), it is believed that the former is likely to adopt similar 3D folding (Li *et al.*, 1996; Gazit *et al.*, 1997).

The sequence of pBtoxis (Berry *et al.*, 2002) identifies a previously unknown gene encoding a putative protein of ~60 kDa (pBt054) with an N-terminal half that is 72 % homologous to Cyt1Aa, hence it was named Cyt1Ca. The C-terminal 280 aa of Cyt1Ca are ~50 % homologous to the β-trefoil modules found in various natural toxins that contain ricin-B-like domains, such as *Clostridium botulinum* neurotoxin, *Pieris brassicae* pierisin-b and the mosquitocidal toxin protein Mtx1 from *Bacillus sphaericus* (Berry *et al.*, 2002). Cyt1Ca is about twice the size of the other Cyt proteins (26–28 kDa), and may represent a novel two-domain fusion toxin.

In this study, *cyt1Ca* and the previously identified *cyt2Ba* were cloned and their products characterized. Their toxicity against larvae of *Aedes aegypti* and their ability to enhance the activity of Cry4Aa were compared with those of Cyt1Aa.

METHODS

Bacterial strains and plasmid vectors. The following plasmids were hosted in *E. coli*: pQE-60 (Qiagen) and its derivative cloned with *cyt1Ca* (Table 1), pGEM-T (Promega), pUHE-24S and its derivatives cloned with *cyt1Ca* (complete and truncated) and *cyt2Ba* (Table 1), pRM4-C containing *cyt1Aa* (Manasherob *et al.*, 2001), and pHE4-A containing *cry4Aa* (Ben-Dov *et al.*, 1995). The vector pGEM-T was introduced into strain BL-21, while the others were hosted in strain XL-Blue MRF′ (Stratagene).

The genes *cyt1Aa*, *cyt2Ba* and *cyt1Ca* were also cloned into the *E. coli–B. thuringiensis* 6·5 kb shuttle vector pHT-315 (Arantes & Lereclus, 1991) under the original *cyt1Aa* promoter. The cloned derivatives of the vector were hosted for expression in the acrystalliferous plasmid-less derivative strain IPS78/11, kindly obtained from D. Ellar (Cambridge, UK). The toxin-coding pBtoxis was isolated as described previously (Ben-Dov *et al.*, 1996) from *B. thuringiensis* subsp. *israelensis* strain 4Q2-72 (kindly supplied by D. R. Zeigler of the Bacillus Genetic Stock Center, Columbus, OH, USA), and was used as a template for PCR amplification of *cyt1Aa*, *cyt2Ba* and *cyt1Ca*.

PCR. The primers used to amplify the three *cyt* genes, the *cyt1Aa* promoter and *p20* from pBtoxis are depicted in Table 1. Taq DNA polymerase (New England BioLabs) was employed for cloning into *E. coli*, and Vent DNA polymerase (New England BioLabs) for cloning into *B. thuringiensis* subsp. *israelensis*, both in a DNA thermal cycler (T-gradient; Biometra) for 30 cycles at the following conditions: 1 min at 94 °C, 50 s at 55 °C and 1–2 min at 72 °C.

Plasmid construction. The blunt-end PCR products (Table 1) were purified from agarose gel by a GFX purification kit (Amersham), digested by the appropriate restriction enzymes (Table 1), and further purified from gels with the same GFX kit. The amplicons for *cyt2Ba* and a different version of *cyt1Ca* were double-ligated into *Nco*I/*Xba*I-digested pUHE-24S or *Nco*I/*Bgl*II-digested pQE-60 for cloning into *E. coli*. For expression in *B. thuringiensis* subsp. *israelensis*, we used either triple ligation of the amplicons for the *cyt1Aa* promoter, with *cyt2Ba* or *cyt1Ca*, into *Sph*I/*Xba*I-digested pHT315, or double ligation of the amplicon of *cyt1Aa* with its own promoter. When *p20* was added, the constructs were subsequently ligated into the *Xba*I–*Sac*I sites of *cyt*-containing pHT315. Verification of all cloned genes was performed by sequencing.

DNA sequences. Sequencing was performed by ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS and the ABI model 373A DNA sequencer system (Perkin–Elmer).

Transformation. The ligated DNA (0·5 μg) was mixed in a 0·2 cm cuvette with a suspension of *E. coli* XL-1 Blue MRF′, and introduced into the bacteria by electroporation (using a Bio-Rad mini apparatus set) at 2·5 kV and 186 Ω. Cloned derivatives of shuttle vector pHT-315 were further electroporated into the acrystalliferous strain IPS78/11 of *B. thuringiensis* subsp. *israelensis*. Screening for transformants was performed on Luria–Bertani (LB) plates, with either 100 μg ampicillin ml^{-1} at 37 °C (for *E. coli*) or 20 μg erythromycin ml^{-1} at 30 °C (for *B. thuringiensis* subsp. *israelensis*).

Gene expression. Cultures of *E. coli* were grown at 37 °C in LB medium supplemented with 100 μg ampicillin and 10 μg tetracycline ml^{-1}, and induced by IPTG (0·5 mM) at OD_{600} 0·2–0·3 ($\sim 2\times10^8$ cells ml^{-1}). Cultures of *B. thuringiensis* subsp. *israelensis* were grown overnight in 5 ml LB medium, transferred to 500 ml CCY sporulation medium (Stewart *et al.*, 1981), with 20 μg erythromycin ml^{-1} at 30 °C for 4 days (Nisnevitch *et al.*, 2006).

E. coli cells and *B. thuringiensis* subsp. *israelensis* spores and crystals were harvested by centrifugation at various times after induction or sporulation, respectively, resuspended in distilled water, and boiled for 10 min in sample treatment buffer (62·5 mM Tris/HCl, pH 6·8, 2 % SDS, 10 % glycerol, v/v, 0·01 % bromophenol blue and 0·1 M DTT) with protease inhibitor PMSF (Sigma P7626; 5 mM). Samples were analysed by SDS-PAGE (Laemmli, 1970). The gels were stained with 0·1 % Coomassie Blue R-250. Protein concentrations were determined with BSA standards (Bradford, 1976).

Western blot analysis. Proteins were electro-transferred from the gel onto nitrocellulose membranes, and exposed to specific antiserum directed against Cyt1Aa (kindly provided by Sarjeet Gill, University of California, Riverside, CA, USA). Protein A–alkaline phosphatase conjugate was the primary antibody detector. Visualization of the antigen was achieved using Sigma Fast 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium tablets (Sigma), the chromogenic substrate for alkaline phosphatase.

Purification of His_6-tagged Cyt1Ca. Purification was performed by nickel-nitrilotriacetic acid (Ni-NTA) column affinity chromatography from the lysate of transgenic *E. coli* cells expressing *cyt1Ca–His*. Cells harbouring pQE-*cyt1CaHis* were harvested after 4 h induction, washed twice, incubated with lysozyme [10 mg (g wet weight of cells)$^{-1}$] for 30 min, and broken up by sonication in 50 mM Tris/HCl (pH 8·0). The mixture was centrifuged, and the supernatant loaded into an Ni-NTA column. Loosely bound proteins were washed from the resin in the above Tris/HCl buffer containing 20 mM imidazole, while the recombinant His_6-tagged Cyt1Ca was eluted by buffer containing 400 mM imidazole, according to the standard procedures of the manufacturer (Qiagen).

Proteolysis of Cyt1Ca–His. Purified Cyt1Ca–His was solubilized at about 1 mg ml^{-1} in 50 mM Na_2CO_3, pH 10·5, at 37 °C for 1 h. Lower pH values were ineffective for solubilization. The solubilized protein was treated with 10 % (w/w) trypsin, chymotrypsin, thermolysin or proteinase K at 37 °C for 1 h.

Haemolytic assay. PBS-suspended sheep erythrocytes were incubated overnight at 37 °C with activated Cyt1Ca (100 μg ml^{-1}), and

Table 1. Primers used in PCR for cloning

Cloned sequence	Orientation	Primer sequence (5′–3′)*	Restriction enzyme	Amplicon size (bp)	Plasmid
cyt1Ca full-length	Forward	CCAGGGGGCGAGT**CCATGG**CTCAATCAATCAGAA	*Nco*I	1618	Cloned to pUHE-24S to get pUH-*cyt1Ca*
	Reverse	GCACTAGTGAT**TCTAGA**CTTTTATTAATTAATC	*Xba*I		
cyt1Ca fused to His-tag coding sequence	Forward	CCAGGGGGCGAGT**CCATGG**CTCAATCAATCAGAA	*Nco*I	1620	Cloned to pQE-60 to get pQE-*cyt1CaHis*
	Reverse	CGCACTAGTGATTCTAGACTT**AGATCT**ATTAATCATGTCC	*Bgl*II		
cyt1Ca truncated at position 722	Forward	CCAGGGGGCGAGT**CCATGG**CTCAATCAATCAGAA	*Nco*I	722	Cloned to pUHE-24S to get pUH-*cyt1Ca*(trE228)
	Reverse	CGGAAG**TCTAGA**TTTAAAATATGCCTCTTAGGGG	*Xba*I		
cyt1Ca truncated at position 744	Forward	CCAGGGGGCGAGT**CCATGG**CTCAATCAATCAGAA	*Nco*I	744	Cloned to pUHE-24S to get pUH-*cyt1Ca*(trF237)
	Reverse	CCCATAAC**TCTAGA**GGTTAATCATGTGTATTTTCG	*Xba*I		
cyt2Ba full-length	Forward	GGGGGATTATTAAA**CCATGG**GCCTTAATAATTTG	*Nco*I	1082	Cloned to pUHE-24S to get pUH-*cyt2Ba*
	Reverse	GAAGTAAAACA**TCTAGA**TACAAAGAGCTCC	*Xba*I		
cyt1Aa promoter	Forward	GGGATTTAGA**GCATGC**TTAAGTAGAATAGACG	*Sph*I	574	Cloned to pHT-315
	Reverse	CAATGATTTAAATTTTC**CATATG**TAAACAACTCC	*Nde*I		
cyt1Aa full-length with its own promoter	Forward	GGGATTTAGA**GCATGC**TTAAGTAGAATAGACG	*Sph*I	1441	Cloned to pHT-315
	Reverse	GTGTGAAGAACAAAT**TCTAGA**GAAAGGGG	*Xba*I		
cyt2Ba full-length	Forward	GGGGGATTATTAA**CATATG**CACC	*Nde*I	858	Cloned to pHT-315
	Reverse	ATCTC**TCTAGA**GTACTATGGCTATTTC	*Xba*I		
cyt1Ca full-length	Forward	CCAGGGGGCGAG**CATATG**GCTCAATCAG	*Nde*I	1708	Cloned to pHT-315
	Reverse	CGGTATCGTCCCT**TCTAGA**ATCAATAC	*Xba*I		
p20 full-length	Forward	CCGTT**TCTAG**AGTAGAAGTCATGTTAGC	*Xba*I	1024	Cloned to pHT-315
	Reverse	CAAATTCATCT**GAGCTC**TTATATCGATTCAC	*Sac*I		

*Bold type indicates restriction recognition sites.

OD_{570} of the supernatant was recorded. Incubation with double-distilled water, PBS or Cyt1Aa was used as a control for haemolytic activity.

Mosquito larvicidal activity. Twenty third- or fourth-instar *Ae. aegypti* larvae, in duplicate, were incubated at 28 °C in 100 ml sterile tap water, with appropriate dilutions of *E. coli* expressing *cyt1Ca*, *cyt2Ba* and *cyt1Aa*. Larval mortality was scored after 24 h. Synergistic interactions between Cyt1Aa, Cyt1Ca, Cyt2Ba and Cry4Aa were tested by feeding with bacterial mixtures in a 1 : 1 ratio by cell number.

Molecular mass determination. The molecular mass of purified His_6-tagged Cyt1Ca, dissolved in a mixture of propanol, double-distilled water and formic acid (2 : 3 : 1, by vol.), was determined using a Reflex IV MALDI-TOF mass spectrometer (Bruker), with an α-cyano-4-hydroxycinnamic acid (CHCA) matrix.

Bacterial viability. Viability was determined by colony-forming ability on LB plates (with 100 μg ampicillin and 10 μg tetracycline ml^{-1}) following appropriate dilutions. The number of colonies was counted after 24 h incubation at 37 °C. Each viability value was calculated from the mean of duplicate values for three different dilutions.

RESULTS AND DISCUSSION

Expressing *cyt1Ca* in *E. coli*, and Cyt1Ca toxicity

The most studied Cyt family toxin, Cyt1Aa, is synergistic with Cry toxins (Crickmore *et al.*, 1995; Khasdan *et al.*, 2001), and delays or prevents the selection for resistance of target insects (Wirth *et al.*, 1997, 2005). Discovering a potentially cytolytic protein from the Cyt family with a binding domain may indicate that it is targeted to the cell via a receptor, as for Cry family toxins. The questions thus arise whether Cyt1Ca is toxic at all; if so, what is its mode of action, and could it be exploited to enhance the biological control of mosquitoes?

To address these issues, *cyt1Ca* was PCR-amplified and cloned into *E. coli* in three vectors, pGEM-T Easy, pUHE-24S and pQE-60, to produce clones designated pGMCB-1C, pUH-*cyt1Ca* and pQE-*cyt1CaHis*, respectively. The recombinant *E. coli* strains were grown in LB medium and induced with 0·5 mM IPTG for 4 h, and the protein content analysed by SDS-PAGE (Fig. 1A). An additional polypeptide, not observed in un-induced cultures, was detected only in cells transformed with the latter two plasmids (Fig. 1A, lanes 2 and 4), but its electrophoretic mobility corresponded to a molecular mass significantly lower (50 kDa) than the expected 60 kDa. Western blot analysis demonstrated that Cyt1Ca did not cross-react with polyclonal anti-Cyt1Aa antibodies (data not shown), despite the 49 % identity between Cyt1Aa and the N-terminal domain of Cyt1Ca.

Interactions of different Cyt toxins with Cry4Aa

The enhancement of Cry4Aa activity by Cyt2Ba and Cyt1Ca has not been reported previously. To compare the ability of the three Cyt proteins of *B. thuringiensis* subsp. *israelensis* to interact with Cry4Aa in this way, *cyt2Ba* was cloned in a similar manner to *cyt1Ca* for expression (see Methods), and the previously cloned *cyt1Aa* (Manasherob *et al.*, 2001) was also used. All three genes (*cyt1Aa*, *cyt2Ba* and *cyt1Ca*) were thus overexpressed in the same *E. coli* strain (XL-Blue MRF′), from the same vector (pUHE-24S), under the control of the same promoter (T7 early), and in the same growth conditions. Each of these was mixed in a 1 : 1 ratio by cell number with clone pHE4-A (expressing *cry4Aa*), and possible synergy in toxicity against *Ae. aegypti* larvae was compared (Fig. 2). No toxicity was displayed by any of these three *cyt*-expressing strains alone at the maximum cell concentration used, 3×10^7 cells ml^{-1} (data not shown). Cry4Aa-producing cells caused 90 % larval mortality at 1×10^7 cells ml^{-1}; this was enhanced to $6{\cdot}3 \times 10^5$ cells ml^{-1} in combination with an equal concentration of Cyt1Aa-producing cells, and to a lesser extent (5×10^6 cells ml^{-1}) in

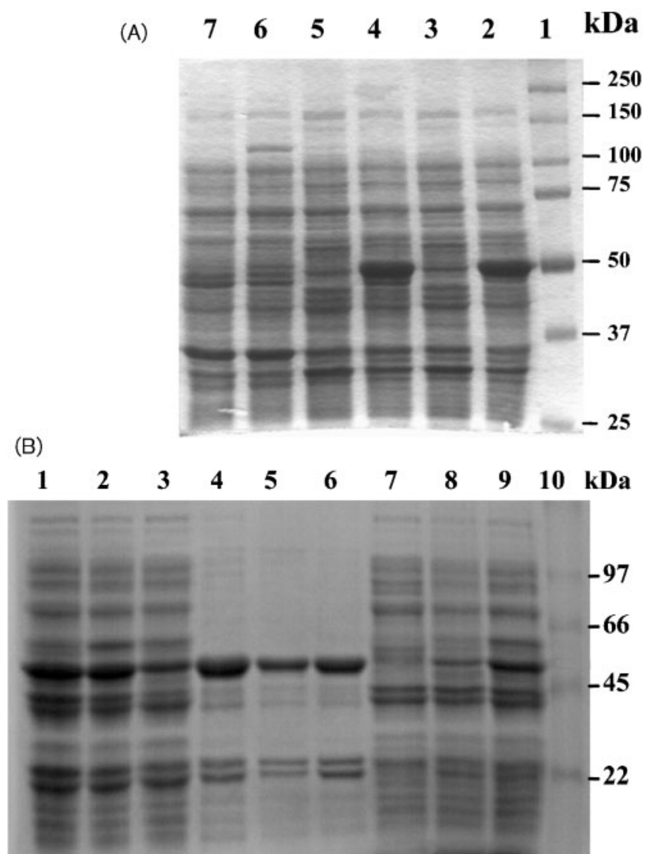

Fig. 1. SDS-PAGE analyses of Cyt1Ca. (A) Extracts of clones (grown and induced at 37 °C) with: pUH-*cyt1Ca* (lanes 2 and 3), pQE-*cyt1CaHis* (lanes 4 and 5), pGCB-1C (lanes 6 and 7); induced (lanes 2, 4 and 6) and un-induced (lanes 3, 5 and 7). Lane 1, protein size marker. (B) Whole extracts (lanes 1–3), pellets (lanes 4–6) and supernatants (lanes 7–9) of the clone with pUH-*cyt1Ca* growing at different temperatures: 37 °C (lanes 1, 4 and 7); 37 °C, heat-shocked for 2 h at 41 °C and continued at 37 °C (lanes 2, 5 and 8); 37 °C, heat-shocked for 2 h at 41 °C and continued at 28 °C (lanes 3, 6 and 9). Lane 10, protein size marker.

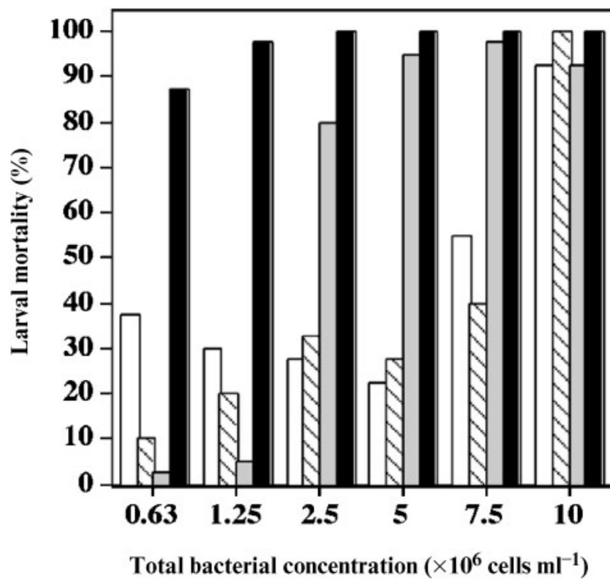

Fig. 2. Mortality of third-instar *Ae. aegypti* larvae fed with cells expressing *cry4Aa* mixed (1 : 1 ratios) with the following clones: pUHE-24S (white bars), pUH-*cyt1Ca* (hatched bars), pUH-*cyt2Ba* (grey bars) and pRM4-C (black bars).

combination with Cyt2Ba-producing cells (Fig. 2). Cyt1Ca-producing cells, however, were unable to supplement the activity of Cry4Aa, even at a ratio of Cyt1Ca : Cry4Aa as high as 4 : 1 (Itsko *et al.*, 2005).

High apparent synergy values between Cyt1Aa and Cry4Aa have previously been demonstrated *in vitro* against larvae of *Ae. aegypti* (Crickmore *et al.*, 1995; Khasdan *et al.*, 2001). Moreover, higher synergy values (between 16·6 and 70·5) were obtained when resistant strains of *Culex quinquefasciatus* were tested, thus demonstrating the significance of Cyt1Aa in suppressing resistance (Wirth *et al.*, 1997). Cyt2Ba has been demonstrated to synergize with *B. sphaericus* (Wirth *et al.*, 2001), but synergy with Cry4Aa has never been demonstrated. The difficulty in expressing *cyt1Aa* in the heterologous host *E. coli* leads to very low levels of production. This makes it hard to compare the synergism of the individual Cyt proteins accurately in these experiments. However, *cyt1Aa*-expressing cells are able to produce significant enhancement of Cry4Aa activity, despite the fact that protein induction can hardly be detected by immunoblotting (Manasherob *et al.*, 2001). In contrast, Cyt2Ba could be visualized by SDS-PAGE (data not shown), yet produced less enhancement of Cry4Aa activity, and Cyt1Ca, also clearly induced in the cultures tested (Fig. 1A, lane 2), produced no effect.

The level of enhancement of Cry4Aa activity by each of the Cyt proteins reflects their relative abundance in *B. thuringiensis* subsp. *israelensis*. Cyt1Aa composes up to 50 % of the crystal, Cyt2Ba is present in very low quantities, and Cyt1Ca is undetectable, although transcript from *cyt1Ca* has been detected (Stein *et al.*, 2006). Perhaps the high-level expression of the most synergistic toxin was selected in this *B. thuringiensis* strain.

In the case of Cyt1Aa, effects on the growth and viability of the heterologous host *E. coli* have been observed within minutes of the induction of protein expression (Manasherob *et al.*, 2001). Production of Cyt1Ca showed no such effects (data not shown), indicating that this protein is not toxic either to mosquito larvae or to *E. coli* cells, although Cyt1Ca mutants have been produced that do display effects on the host bacterium, but not on mosquitoes (Itsko *et al.*, 2005).

Cloning of three *cyt* genes for expression in *B. thuringiensis* subsp. *israelensis*

To rule out the possibility that Cyt1Ca is not properly folded in *E. coli*, and hence loses its presumed activity, the three *cyt* genes, *cyt1Aa*, *cyt2Ba* and *cyt1Ca*, were cloned under the strong *cyt1Aa* promoter in the expression vector pHT315 into acrystalliferous *B. thuringiensis* subsp. *israelensis*. While *cyt1Aa* and *cyt2Ba* displayed substantial expression, *cyt1Ca* did not produce Cyt1Ca at all (Fig. 3). Moreover, the accessory protein P20, known to raise the levels of Cyt1Aa (Wu & Federici, 1993) and of Cyt2Ba (Nisnevitch *et al.*, 2006) in acrystalliferous strains of *B. thuringiensis* subsp. *israelensis*, did not assist in *cyt1Ca* expression (Fig. 3). Similarly, another gene of *B. thuringiensis* subsp. *israelensis*, *p19*, presumed to encode an accessory protein, has been shown to be expressed in *E. coli* (Manasherob *et al.*, 2001) but not in *B. thuringiensis* (unpublished data). Transcripts of *cyt1Ca* and *cyt2Ba* have recently been detected in strain 4Q5

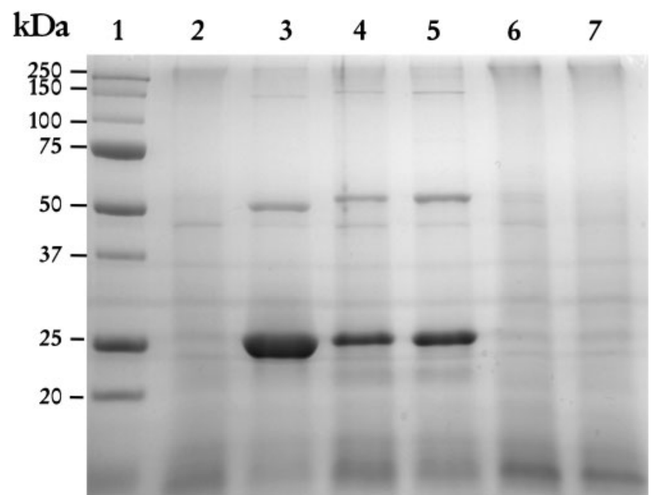

Fig. 3. SDS-PAGE analysis of Cyt-like proteins produced in *B. thuringiensis* subsp. *israelensis*. Extracts of clones with pHT-315 (lane 2), pHT-*cyAp20* (*cyt1Aa*+*p20*) (lane 3), pHT-*cyB* (*cyt2Ba*) (lane 4), pHT-*cyBp20* (*cyt2Ba*+*p20*) (lane 5), pHT-*cyC* (*cyt1Ca*) (lane 6), and pHT-*cyCp20* (*cyt1Ca*+*p20*) (lane 7) are shown.

(Stein *et al.*, 2006), without their products; this observation is explained by instability of the transcript or of the resultant protein, or failure in translation. Here, replacing the original respective promoters with that of *cyt1Aa* resulted in high production of Cyt2Ba but none of Cyt1Ca. Consistently, haemolytic activity was detected in the former clone (Nisnevitch *et al.*, 2006) but not in the latter (data not shown).

Characterization of Cyt1Ca

The possibility that Cyt1Ca activity is neutralized by aggregation into inclusion bodies was considered. Most of the Cyt1Ca in *E. coli* cells (expressed from pUH-*cyt1Ca* during growth at 37 °C) was indeed found in the pellet (Fig. 1B, lane 4); thus, the low concentration of soluble Cyt1Ca may have been a reason for the lack of lethal activity. To test this possibility, the cells were heat-treated (41 °C for 2 h) before induction with IPTG to raise the quantity of chaperones (Baneyx, 1999). Higher levels of soluble Cyt1Ca were thus obtained (Fig. 1B, compare lanes 8 and 7). The amount was even greater when cells were transferred to 28 °C after induction (Fig. 1B, compare lane 9 with 8 and 7). Nevertheless, no change in either toxicity to the host bacterium or larvae, or in the ability to enhance the activity of Cry4Aa, was observed in bioassays repeating those described in Fig. 2 (data not shown).

For further characterization of Cyt1Ca, *cyt1Ca* was cloned into pQE-60 to encode a fusion protein with six His residues at the C terminus, and the chimera was purified on a Ni-NTA column. The SDS-PAGE mobility of the purified polypeptide was indeed significantly lower (~50 kDa) than the 60 kDa predicted from the gene sequence (Fig. 4, inset). The sequence of the *cyt1Ca* gene in this clone was verified to rule out mutation during PCR amplification, and was identical to the published sequence (Berry *et al.*, 2002). Sequencing the N-terminal amino acids (MAQSEF) confirmed that the protein was Cyt1Ca, and analysis by MALDI-TOF MS yielded a size of 61·259 kDa (Fig. 4), almost exactly that (61·398 kDa) of the chimeric Cyt1Ca–His_6. Thus, the lack of toxicity was not a result of Cyt1Ca degradation while expressed in *E. coli*. The faster migration of Cyt1Ca in SDS-PAGE thus seems to derive from some unusual intrinsic structure.

The purified Cyt1Ca was assayed for haemolytic activity on sheep erythrocytes (Fig. 5), but no significant effect was detected. Proteolytic activation of Cyt1Ca with proteinase K, chymotrypsin and trypsin yielded only a marginal effect (Fig. 5), confirming that Cyt1Ca is neither bacteriolytic (when expressed in *E. coli*) nor cytolytic (when added to larvae and erythrocytes). The low haemolytic activity observed may have resulted from the high pH (10·5) used for solubilization, but the Cyt proteins did not dissolve at the pH range (7–9) optimal for the proteases, a pH range at which Cyt proteins precipitate (Du *et al.*, 1999).

It is possible that the lack of toxicity of Cyt1Ca is connected to the β-trefoil module in its C-terminal region. This ricin B-like domain may interfere with the insertion and organization of the Cyt-like part of Cyt1Ca into the membrane of target cells. Cyt1Aa loses its lethal activity when fused at its C terminus to green fluorescent protein (GFP) (Manasherob *et al.*, 2003), indicating that the organization of the C terminus of Cyt1Aa may be crucial for its activity. In

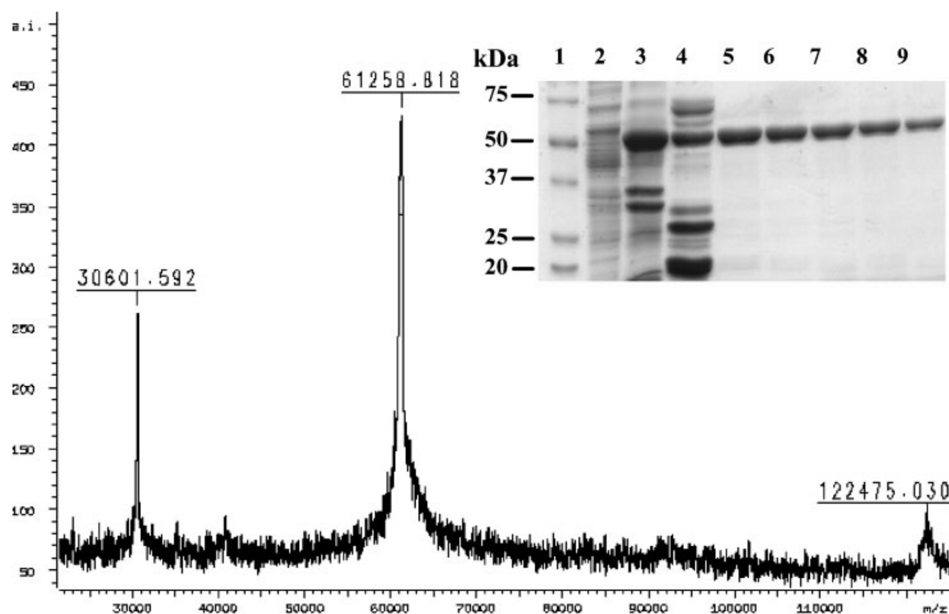

Fig. 4. MALDI-TOF mass spectrum of purified His_6-tagged Cyt1Ca. Inset, SDS-PAGE analysis: supernatant (lane 2) and pellet (lane 3); elution fractions (lanes 4–9). Lane 1, protein size marker.

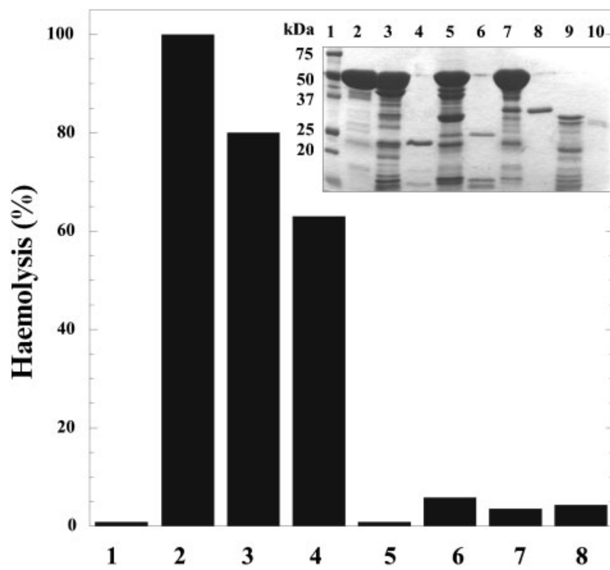

Fig. 5. Haemolysis of sheep erythrocytes. Erythrocytes were incubated with a Cyt1Ca–HisTag (100 μg ml^{-1}), untreated (column 5) or treated with chymotrypsin (column 6), trypsin (column 7) or proteinase K (column 8). Column 1, in PBS; column 2, in double-distilled water; column 3, extract from *B. thuringiensis* subsp. *israelensis* spores (pH 10·5); column 4, Cyt1Aa purified from *B. thuringiensis* subsp. *israelensis* (2 μg ml^{-1}). Inset, proteolysis of Cyt1Ca–HisTag. The solubilized Cyt1Ca–HisTag was untreated (lane 2), or treated with trypsin (lane 3), chymotrypsin (lane 5), thermolysin (lane 7) or proteinase K (lane 9). The corresponding proteases were run next to the treated Cyt1Ca–HisTag (lanes 4, 6, 8 and 10) Lane 1, molecular mass marker.

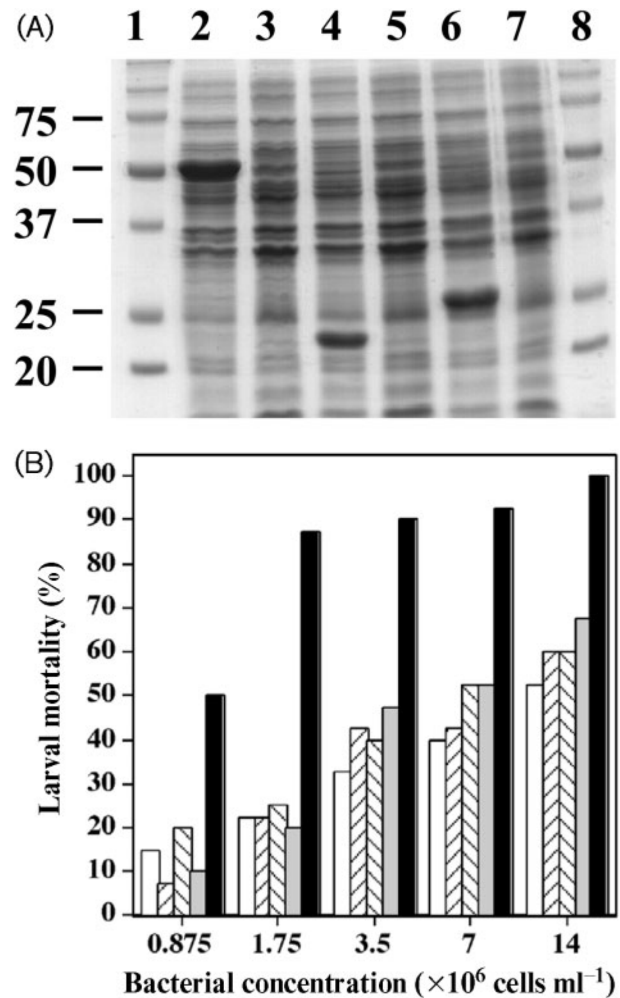

Fig. 6. (A) SDS-PAGE analysis of whole extracts of clones. Clones contained: pUH-*cyt1Ca* (full-length Cyt1Ca) (lanes 2 and 3); pUH-*cyt1Ca*(trE228) (Cyt1Ca truncated at Glu 228) (lanes 4 and 5); pUH-*cyt1Ca*(trF237) (Cyt1Ca truncated at Phe 237) (lanes 6 and 7); induced (lanes 2, 4 and 6) or un-induced (lanes 3, 5 and 7). Lanes 1 and 8, protein size markers. (B) Mortality of fourth-instar *Ae. aegypti* larvae fed with cells expressing *cry4Aa* mixed (1 : 1 ratios) with the following clones: pUH-*cyt1Ca* (white bars); pUH-*cyt1Ca*(trF237) (hatched bars second from left); pUH-*cyt1Ca*(trE228) (centre hatched bars); pUHE-24S, vector only control (grey bars); and pRM4-C-producing Cyt1Aa (black bars).

attempts to obtain a toxic variant of Cyt1Ca, the 3′ end of *cyt1Ca* was genetically truncated at positions 722 and 744 bp (to generate proteins truncated at E228 and F237, respectively) (Table 1, Fig. 6A), to mimic Cyt1Aa and Cyt2Aa proteinase K activation sites (Al-Yahyaee & Ellar, 1995; Du *et al.*, 1999). A new set of bioassays was performed with the truncated clones using fourth-instar mosquito larvae (Fig. 6B), but no significant differences in mortality (Itsko *et al.*, 2005) or synergism with cry4Aa-expressing cells were observed with the truncated Cyt1Ca.

It is interesting to speculate that Cyt-like toxins have evolved from a Cyt1Ca-like fusion protein by loss of the β-trefoil domain, or that Cyt1Ca has acquired such a domain, after the evolution of Cyt toxins. In the case of *cyt1Aa*, a convergent coding sequence (pBt020) exists on pBtoxis (accession no. AL731825) and is separated from *cyt1Aa* by only 2 nt, a situation indicating genetic rearrangement in this region that could have deleted an ancestral C-terminal domain. However, this feature of a close, convergent coding sequence does not appear to occur with other *cyt* sequences. The facts that Cyt1Aa is adversely affected by C-terminal fusions (Manasherob *et al.*, 2003), and that Cyt1Ca appears inactive, indicate that the ancestor is rather of the single Cyt domain type.

In summary, a novel protein, Cyt1Ca from *B. thuringiensis* subsp. *israelensis*, has been characterized. This protein was composed of two domains, Cyt- and lectin-like, suggesting a receptor-binding ability not recognized in any previously known Cyt protein. However, the protein and its genetically engineered truncated version were neither toxic nor able to enhance the toxicity of Cry4Aa, in contrast to the other Cyt proteins, Cyt2Ba and Cyt1Aa.

ACKNOWLEDGEMENTS

This investigation was partially supported by a grant (no. 2001-042) from the United States–Israel Binational Science Foundation (BSF), Jerusalem, Israel. We thank Yoel Margalit for the generous supply of *Ae. aegypti* eggs.

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